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# Study on Selective Media for Isolation of Entomopathogenic Fungi

**Tae-Young Shin, Jae-Bang Choi, Sung-Min Bae, Hyun-Na Koo and Soo-Dong Woo***

*Department of Agricultural Biology, College of Agriculture Life & Environment Science, Chungbuk National University, Cheongju 361-763, Korea*

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**To determine the optimal isolation conditions of the entomopathogenic fungi from soil, we compared their growth characteristics with non-entomopathogenic fungi on agar media containing various concentrations of cooper (II) chloride ($CuCl_2$) or dodine. The result showed that dodine medium is more selective, and the optimal concentration of dodine is determined with 50 μg/ml. We could isolate several putative entomopathogenic fungi from soil using this, and identify them using ITS analysis. As a result, 64% fungi were identified as typical entomopathogenic fungi. This selective medium may be useful to the rapid and simple isolation of entomopathogenic fungi from soil.**

**Key words:** Entomopathogenic fungi, Dodine, Selective medium

## Introduction

In agricultural fields, the entomopathogenic fungal species have been investigated for their potential as the biological control agents due to their role of natural enemies for insects. Conidia that adhere to the surface of the host release extracellular enzymes including lipases, proteases and chitinase that help breach the host's chitinous exoskeleton (Freimoser *et al*., 2003; Pendland *et al*., 1993; Tscharntke *et al*., 2005). These fungi have been documented to occur naturally in over 750 species of host and development of microbial insecticide in advanced countrys (Hejek and St. Leger, 1994; Inglis *et al*., 2001; Shah and Pell, 2003). Until recent times, most of the studies for these fungi have been based on isolation from cadavers of insect or soil (Abdo *et al*., 2008; Brownbridge *et al*., 2010; Glare *et al*., 2008; Santoro *et al*., 2008; Vu *et al*., 2007). In previous reports, both two methods with a susceptible insect host and selective media have been utilized to the isolation of entomopathogenic fungi from soil (Shimazu and Sato, 1996; Veen and Ferron, 1966; Zimmermann, 1986, 1998). Use of insect bait is a very sensitive detection method and entomopathogenic fungi can be selectively isolated. However, some insect species may select for specific fungal pathogens and difficult to quantify inoculums levels. Although the isolated fungi must be evaluated its pathogenicity to target insect, by contrast, selective media have some advantages for the mass collection of positive entomopathogenic fungi and quantitative data. Therefore, various selective media have been developed for the mass collection of entomopathogenic fungi from soil (Meyling, 2007). Recently, several fungicides (such as oxgall, cupric sulfate, cooper (II) chloride ($CuCl_2$), benomyl and dodine) and antibiotics (such as cholramphenicol, tetracycline and streptomycin) have been used separately or in combination to detect various entomopathogenic fungi on selective media (Beilhartz *et al*., 1982; Chase *et al*., 1986; Keller *et al*., 2003; Mark and Douglas, 1997; Meyling and Eilenberg, 2006; Shimazu and Sato, 1996; Shimazu *et al*., 2002). Among them, dodine and $CuCl_2$ have been evaluated as more effective to the isolation of entomopathogenic fungi from soil (Chase *et al*., 1986; Shimazu and Sato, 1996). Therefore, the objective of this study was development of the optimal selective medium using these for the mass collection and isolation of potential Korean based entomopathogenic fungi from soils.

## Material and Methods

### Fungi

All entomopathogenic fungi were obtained from Korean

*To whom the correspondence addressed
Department of Agricultural Biology, College of Agriculture Life & Environment Science, Chungbuk National University, Cheongju 361-763, Korea.
Tel: +82-043-261-2553; Fax: +82-043-271-4414;
E-mail: sdwoo@cbnu.ac.kr

**Table 1.** Fungal strains used in this study

| Strain | Species | Characteristics |
|---|---|---|
| SFB-13 | *Beauveria Bassiana* | Entomopathogen |
| MaW1 | *B. Bassiana* | Entomopathogen |
| MsW1 | *B. Bassiana* | Entomopathogen |
| F-1031 | *B. Bassiana* | Entomopathogen |
| KACC 40216 | *B. Bassiana* | Entomopathogen |
| KACC 40224 | *B. Bassiana* | Entomopathogen |
| KCTC 6300 | *B. Brogniartii* | Entomopathogen |
| KACC 40502 | Cordyceps militaris | Entomopathogen |
| KACC 40226 | C. militaris | Entomopathogen |
| KACC 40023 | Hisutella thompsonii | Entomopathogen |
| KACC 40029 | M. anisopliae | Entomopathogen |
| KACC 40217 | *Metarhizium sp.* | Entomopathogen |
| KACC 40223 | *Metarhizium sp.* | Entomopathogen |
| KACC 40503 | *Isaria fumosoroseus* | Entomopathogen |
| KACC 40220 | Isaria sp. | Entomopathogen |
| KACC 40220 | Isaria sp. | Entomopathogen |
| SFP-198 | *I. fumosoroseus* | Entomopathogen |
| ARSEF 1014 | Nomurae rileyi | Entomopathogen |
| GKPE-1 | Alternaria alternata | Non-entomopathogen |
| GBYP-8 | Botrytis cinerea | Non-entomopathogen |
| JC-24 | Colletotrichum acutatum | Non-entomopathogen |

Agricultural Culture Collection (KACC), Korean Collection for Type Cultures (KCTC) and the insect microbiology & biotechnology laboratory of Seoul National University except for *Beauveria bassiana* MaW1 (Shin *et al*., 2009) and *B. bassiana* MsW1 isolated from *M. saltuarius* Gebler (Coleoptera: Cerambycidae) was obtained and identified from our own culture. Non-entomopathogenic fungi were obtained from the plant fungal disease laboratory of Chungbuk National University, Korea. All fungal strains are listed in Table 1. Fungi were cultured on PDA media at 25°C in dark condition.

**Selective media**

To determine the optimal selective medium for entomopathogenic fungi, various media were prepared by the modifying previously reported D0C2 medium (Shimazu and Sato, 1996) and Veens semiselective medium (Veen and Ferron, 1996). Information of media used in this study is presented in Table 2. Selectivity of medium was determined by comparing growth rate.

**Isolation of entomopathogenic fungi from soil**

A 0.2 g amount of soil sample was placed in a 1.5 ml micro tube with 1.3 ml of 0.02% Tween-80 solution and vortexed for 15 min. The resulting suspension was serially diluted ($10^{-1}$) and plated on selective medium. After incubation for 6 days at 25°C, the putative entomopathogenic fungi were selected by morphological characteristics (aspects of the colonies, such as color, diameter and mycelia texture).

**Table 2.** Various selective medium for entomopathogenic fungi used in this study

| Selective medium | Consisted |
|---|---|
| D0C2-10 | 3 g Bactopeptone, 0.2 g $CuCl_2$, 2 mg crystal violet, 15 g agar, 1,000 ml distilled water, pH 10.0 with $Na_2CO_3$ |
| D0C2-4 | 3 g Bactopeptone, 0.2 g $CuCl_2$, 2 mg crystal violet, 15 g agar, 1,000 ml distilled water, pH 4.0 with HCl |
| D0C2-50% | 3 g Bactopeptone, 0.1 g $CuCl_2$, 2 mg crystal violet, 15 g agar, 1,000 ml distilled water, pH 4.0 with HCl |
| D0C2-PDA | 0.2 g $CuCl_2$, 2 mg crystal violet, 39 g PDA (potato dextrose agar), 1,000 ml distilled water, pH 4.0 with HCl |
| SM | 10~100 μg/ml Dodine, 100 μg/ml Chloramphenicol, 50 μg/ml Streptomycin, 32.5 g SDA (sabouraud dextrose agar) in 500 ml distilled water. |

**Genomic DNA isolation**

Fungal genomic DNA was extracted from the hyphae using a partially modified chemical lysis method (St. Leger and Wang, 2009). The fungal isolate was inoculated into 1.5 ml micro tube with PD (potato dextrose broth) and incubated for 3~4 days at 25°C on a shaker at 250 rpm. The mycelia was pelleted by centrifuging at 10,000 rpm for 10 min and resuspended in 400 μl of fungal DNA extraction buffer (0.2 M Tris-Cl (pH 7.5), 0.5 M NaCl, 10 mM EDTA (pH 8.0) and 1% (w/v) SDS). Then 400 μl of phenol-chloroform-isoamylalcohol (25:24:1) was added, and the mixture was vortexed for 5 min. After centrifugation at RT/10,000 rpm for 8 min, the aqueous upper layer was transferred to a new microcentrifuge tube. After adding 1 μl of RNase solution (20 mg/ml, Sigma) into the sample, it was incubated at 37°C for 30 min and purified again with a phenol-chloroform-isoamylalcohol (25:24:1) as previously described. The DNA in the aqueous phase was precipitated with 2.5 volume of 100% ethanol. The sample was centrifuged at 4°C/12,000 rpm for 10 min and the pellet was washed with 70% ethanol, dried and resuspended in 50 μl of $ddH_2O$. The extracted DNA solution was used as a template for PCR.

**Table 3.** Effect of various selective medium on fungi

| Selective medium | | | D0C2-10 | D0C2-4 | D0C2-PDA | D0C2-50% | SM (100 μg/ml dodine) |
|---|---|---|---|---|---|---|---|
| Strain | Species | Characteristics[a] | | | | | |
| GKPE-1 | *A. alternata* | PP | - | - | - | - | - |
| GBYP-8 | *B. cinerea* | PP | - | - | +++ | + | - |
| JC-*24* | *C. acutatum* | PP | - | - | +++ | + | +/- |
| MaW1 | *B. bassiana* | EP | - | - | - | - | + |
| MsW1 | *B. bassiana* | EP | - | - | +++ | +++ | + |
| SFB-13 | *B. bassiana* | EP | - | - | +++ | +++ | ++ |
| F-1032 | *B. bassiana* | EP | - | - | - | - | +/- |
| KCTC 6300 | *B. brogniartii* | EP | - | - | - | | ++ |
| SFP-198 | *I. fumosoroseus* | EP | ++ | ++ | +++ | + | ++ |
| KACC 40217 | *M. anisopliae* | EP | - | - | +++ | +++ | + |
| ARSEF 1014 | *N. releyi* | EP | | | | +++ | ++ |

[a]PP, plant pathogenicity; EP, entomopathogenicity
-, no growth; +/-, very low; +, low; ++, middle; +++, high

**PCR and ITS analysis**

To amplify the internal transcribed spacers (ITS1-5.8S-ITS2) region, ITS1-forward (5'-TCCGTAGGTGAACCT-GCGG-3') and ITS4-backward (5'-TCCTCCGCTTAT-TGATATGC-3') primers were synthesized following previous report (White *et al*., 1990). The PCR reaction mixture consisted of AccuPOWER$^{TM}$ PCR PreMix (Bioneer Co., Korea), 1 μl of DNA solution and 10 pmol of each primer in a 20 μl volume. The reaction parameters were as follows: initial denaturation for 5 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and a final 10 min extension at 72°C using a thermal cycler (Takara, Japan). After PCR, the amplified DNA fragments were separated by electrophoresis in 1.0% agarose gel and extracted by using a Power Gel Extraction Kit (Dyne Bio Inc., Korea). The purification PCR products used directly sequencing (Sol-Gent Co. Korea). Sequences were aligned using DNA-MAN (Lynnon BioSoft, Quebec, Canada). The obtained sequences of the ITS region was compared with other fungi using the BLAST search tool.

## Result and Discussion

**Determination of selective medium**

The growth rates of entomopathogenic and non-entomopathogenic fungi were compared on various selective media for isolation of entomopathogenic fungi. The results showed that more entomopathogenic fungi and inhibition of non-entomopathogenic can be grown on dodine contained medium than on $CuCl_2$ contained (Table 3). Although tested some entomopathogenic fungi could grew on selective media D0C2-PDA and D0C2-50% containing $CuCl_2$, they permitted the growth of non-entomopathogenic fungi. In previous reports, dodine have been successfully used to isolate *Beauveria* spp. and *Metarhizium* spp. from soil and cadavers of insect. An agar medium amended with 650 μg/ml dodine suppressed growth of non-entomopathogenic fungi however supported growth of entomopathogenic fungi (Beilhartz *et al*., 1982). Subsequently, a medium amended with 600 μg/ml of dodine resulted in good isolation of *B. bassiana*. However this concentration of dodine was inhibitory to *M. anisopliae* (Chase *et al*., 1986). When the concentration was reduced to 500 μg/ml of dodine and 400 μg/ml of the benomyl was added to medium, both *B. bassiana* and *M. anisopliae* were effectively isolated from soil. The use of dodine for isolation of entomopathogenic fungi has relied solely on empirical observations at various laboratories. The selective growth of entomopathogenic fungi could be observed on SM media containing dodine from 60 to 100 μg/ml, but it was hard (Table 3). To determine the optimal concentration of dodine, therefore, we further investigated the selectivity of SM media by reducing dodine concentrations. The result showed that the concentrations below 40 μg/ml of dodine allow good growth of almost tested entomopathogenic fungi, but also allow it to non-entomopathgenic fungi (Table 4). The optimal concentration of dodine was determined with 50 μg/ml and this medium was named SDA-D50 (Table 4) (Fig. 1).

**Isolation of entomopathogenic fungi using SDA-D50**

The selectivity of SDA-50 medium for the entomopatho-

**Table 4.** Effect of various concentrations of dodine on fungi

| Selective medium | | | SM 50 mg/ml dodine | SM 40 mg/ml dodine | SM 30 mg/ml dodne | SM 20 mg/ml dodine | SM 10 mg/ml dodine |
|---|---|---|---|---|---|---|---|
| Strain | Species | Characteristics | | | | | |
| GKPE-1 | *A. alternata* | PP | - | + | + | + | + |
| GBYP-8 | *B. cinerea* | PP | - | + | + | + | + |
| JC-*24* | *C. acutatum* | PP | +/- | + | + | + | + |
| MaW1 | *B. bassiana* | EP | ++ | ++ | N/D | N/D | N/D |
| MsW1 | *B. bassiana* | EP | ++ | ++ | N/D | N/D | N/D |
| SFB-13 | *B. bassiana* | EP | +++ | +++ | N/D | N/D | N/D |
| F-1032 | *B. bassiana* | EP | + | + | N/D | N/D | N/D |
| KCTC 6300 | *B. brogniartii* | EP | ++ | ++ | N/D | N/D | N/D |
| SFP-198 | *I. fumosoroseus* | EP | +++ | +++ | N/D | N/D | N/D |
| KACC 40217 | *M. anisopliae* | EP | ++ | ++ | N/D | N/D | N/D |
| ARSEF 1014 | *N. releyi* | EP | +++ | +++ | N/D | N/D | N/D |

PP, plant pathogenicity; EP, entomopathogenicity
-, no growth; +/-, very low; +, low; ++, middle; +++, high
N/D, not determined

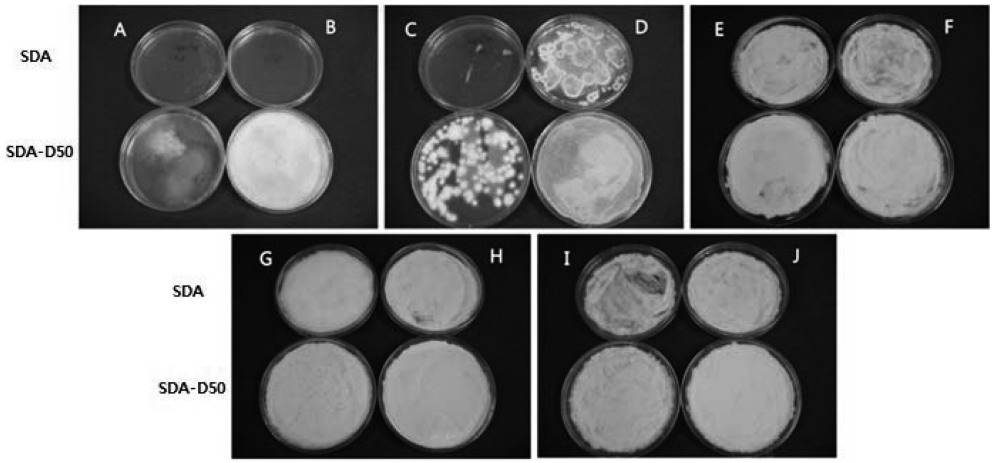


**Fig. 1.** Comparison of the growth between entomopathogenic fungi and non-entomopathogenic fungi on SDA-D50 medium at 5 days. A, GKPE-1; B, GBY-98; C, MaW1; D, MsW1; E, F-1032; F, SFB-13; G, KCTC6300; H, KACC40217; I, SFP-198 and J, ARESF 1014.

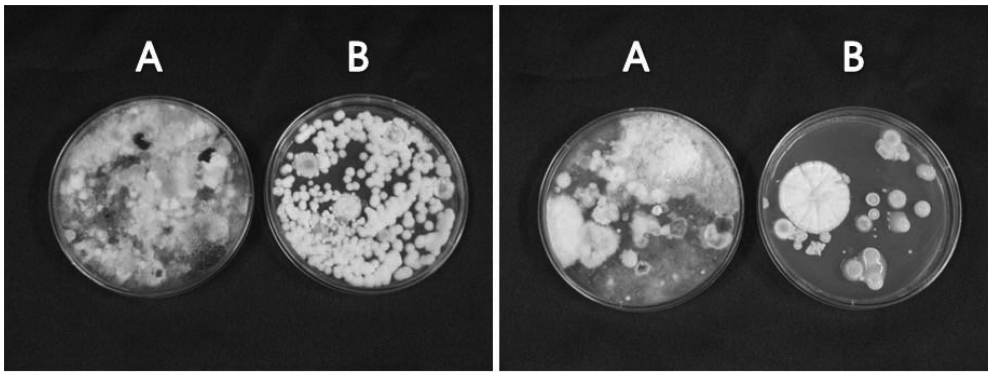


**Fig. 2.** Comparison of microorganism growth including putative entomopathogenic fungi from soil samples. A, SDA medium; B, SDA-D50 medium.

**Table 5.** Identification of isolated fungi using SDA-D50 from soil

| Strain | Identification | Characteristics[a] |
|---|---|---|
| DK-R01-W1 | Beauveria bassiana | EP |
| JN10-R02-W1 | Pochonia bulbillosa | EP |
| JN15-F04-G1 | Scopulariopsis brevicaulis | NP |
| KW18-G03-W1 | Isaria javanicus | EP |
| KB03-G03-G1 | *Isaria* sp. | EP |
| KB03-G04-W1 | I. marquandii | EP |
| JN01-M02-W1 | B. bassiana | EP |
| JN01-M02-W1 | *Cordyceps* sp. | EP |
| KN10-M01-W1 | *Bionectria ochroleuca* | NP |
| JN05-T08-W1 | Chaunopycnis alba | NP |
| JN19-T01-W1 | Chaunopycnis alba | NP |
| DK06-T06-W1 | Chaunopycnis alba | NP |
| JN10-T03-W1 | Fusarium oxsporum | EP |
| KN02-S01-W1 | *Penicillium* sp. | NP |
| JB04-S03-W1 | I. javanicus | EP |
| JN13-S05-W1 | B. bassiana | EP |
| JN15-S05-W1 | I. javanicus | EP |

[a]EP, entomopathogenicity; NP, non-entomopathogenicity

genic fungi was tested to soil samples. SDA-D50 medium permitted virtually growth of putative entomopathogenic fungi while strongly inhibiting many non-entomopathogenic fungi and bacteria but some of contamination with members of the actinomycetes when comparing with SDA medium (Fig. 2). This indicated that SDA-D50 medium can be used well to isolation of entomopathogenic fungi from soil (Fig. 2).

**Identification of fungi**

To identify putative entomopathogenic fungi isolated from soil, ITS regions were amplified and sequenced. Eleven of analyzed 17 fungi were identified as typical entomopathogenic fungi including *Beauveria bassiana* (Table 5). This suggests that the SDA-D50 medium support 64% selection ability of entomopathogenic fungi from soil and One of six fungi to known non-putative entomopathogenic fungi *Penicillium* sp. has evaluative as entomopathogenic fungi in several reports (Da Costa *et al*., 1998; Srivastava *et al*., 1994).

The importance of securing resource of entomopathogenic fungi is increasing steadily because their high potential as the biological control agents and other various applications. Therefore, this isolation method described may be useful for rapid and simple isolation of entomopathogenic fungi from soil. This is the first record of isolation of entomopathogenic fungi using dodine in Korea.

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